# Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Ewing CM, Ray AM, Lange EM, et al. Germline mutations in *HOXB13* and prostate-cancer risk. N Engl J Med 2012;366:141-9.

**Supplementary Appendix**

Supplement to Ewing CM, Ray AM, Lange EM et al. Germline mutations in *HOXB13* and prostate cancer risk

**Contents**

**Supplementary Methods: Targeted Sequencing**

A primer library was designed for amplification of ~2800 amplicons representing 2009 exons from our target region. We then used the RainDance RDT 1000 system (RainDance Technologies, Inc., Lexington MA) to amplify 3 ug of sheared genomic DNA from each sample using our primer library. Purified amplicons were used as template for sequencing using the Life Technologies SOLiD™ system, version 4.0 fragment library methodology (Life Technologies Corporation, Carlsbad, CA). Sequence data processing was performed using Life Technologies Bioscope to align the sequences to the genomic reference (Build 36, hg18). Variant detection was performed using SamTools 1.3[1] and SolSNP 1.1. We confirmed and tested all variant sequences in family members using standard Sanger sequencing, capillary electrophoresis technology and BigDye® Terminator chemistry (Applied Biosystems, Carlsbad CA).

**Supplementary Results: Sequencing Statistics**

We sequenced 2009 exons from genes in our chromosome 17 candidate interval. The average depth of coverage across all loci was 49.5X. There were a total of 20 loci that yielded an average depth of coverage <1X (<1%), while 2040 loci had an average depth of coverage >10X (97%). We detected on average 705 variants per sample across our target region. Approximately 694 variants on average were present in dbSNP134 (98%), with an average of ~12 novel variants per individual.

**Previously identified prostate cancer SNPs on chromosome 17q and *HOXB13***

There are three chromosome 17q loci that have been implicated in prostate cancer susceptibility: 17q12 (rs4430796, HNF1B), 17q21 (discussed below) and 17q24 (rs1859962).[2] The 17q12 and 17q24 loci are over 10 and 20 Mb, respectively, from *HOXB13* and therefore likely independent. However, the 17q21 SNP identified by Haiman et al.,[3] is within 1Mb from *HOXB13* and additional experiments were conducted to uncover a possible relationship between these two genetic loci.

Since the 17q21risk allele is uniquely identified in African American men, we focused on studies in this population.

Recently, Haiman et al.[3] observed an association between a SNP at 17q21 and prostate cancer risk in men of African descent. This risk locus (rs7210100) lies ~630kb telomeric of *HOXB13*. To explore a potential relationship between rs7210100 and *HOXB13* variants, we sequenced both exons of *HOXB13* in 24 African American male carriers of the rs7210100 risk associated allele (4 homozygous carriers and 20 heterozygotes). All 24 men, including12 prostate cancer cases (2 homozygotes and 10 heterozygotes) and 12 prostate cancer free controls, were included in the original report by Haiman et al. We failed to identify any rare *HOXB13* mutations in either the cases or controls that were either homozygous or heterozygous for the rare cancer associated allele at rs7210100. This data, while preliminary in scope, suggests that rs7210100 and *HOXB13* variants play an independent role in prostate cancer susceptibility in African Americans.

**HOXB13 mutations and prostate cancer in African Americans**

It is unclear how important HOXB13 variants, especially G84E, are with respect to prostate cancer risk in African Americans. We did not observe any G84E carriers among 91 unrelated prostate cancer cases of African descent. As reported by the ESP Exome Variant Server,[4] only 2/933 (frequency = 0.0021) African American subjects were reported to carry the G84E variant “T” allele. Age and gender are not reported in the Exome Variant Server and thus it is not clear if the two carriers were adult males. We note that we did observe two missense variants at highly-conserved amino acid residues (neither of which were reported by ESP at the time of our manuscript submission or observed among our subjects of European descent) among these 91 prostate cancer cases of African descent, suggesting there may be *HOXB13* risk variants that are unique to this population. Larger sample sizes of African American men with prostate cancer will be required to have sufficient

statistical power to evaluate the association between *HOXB13* variants and prostate cancer in this population.

**REFERENCES:**


1. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078-9.

2. Gudmundsson J, Sulem P, Steinthorsdottir V, et al. Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. NatGenet 2007;39:977-83.

3. Haiman CA, Chen GK, Blot WJ, et al. Genome-wide association study of prostate cancer in men of African ancestry identifies a susceptibility locus at 17q21. Nature Genetics 2011;43:570-3.

4. Exome Variant Server. NHLBI Exome Sequencing Project (ESP);Seattle, WA (URL: http://snp.gs.washington.edu/EVS/).


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**Supplementary Table 1 Complete *HOXB13* Sequencing Results form 94 Men with Prostate Cancer**
*HOXB13* variants identified in 85 Caucasian samples

| Chrm 17 Location* | Ref Allele/Variant Allele | dbSNP ID | Variant Type | Substitution | # Individuals [N(%)] |
|---|---|---|---|---|---|
| 44157313 | G/T | rs116931900** | 3' UTR | NA | 7 (8.2) |
| 44157328 | T/C | rs79812861** | 3' UTR | NA | 5 (5.9) |
| 44157843 | G/C | novel | 3' UTR | NA | 1 (1.2) |
| 44158537 | C/T | rs11653611 | 3' UTR | NA | 51 (60.0) |
| 44160442 | A/G | rs9900627 | Synonymous | S171S | 13 (15.3) |
| 44160589 | G/A | rs8556 | Synonymous | S122S | 20 (23.5) |
| 44160704 | C/T | rs138213197** | Nonsynonymous | G84E | 4 (4.7) |
| 44161086 | T/C | novel | 5' UTR | NA | 2 (2.4) |

*HOXB13* variants identified in 7 African American samples

| Chrm 17 Location* | Ref Allele/Variant Allele | dbSNP ID | Variant Type | Substitution | # Individuals [N(%)] |
|---|---|---|---|---|---|
| 44157328 | T/C | rs79812861** | 3' UTR | NA | 1 (14.3) |

| | | | | | |
|---|---|---|---|---|---|
| 44158537 | C/T | rs11653611 | 3' UTR | NA | 2 (28.6) |
| 44159094 | C/T | rs141179592** | 3' UTR | NA | 1 (14.3) |
| 44159321 | G/C | novel | Nonsynonymous | R229G | 1 (14.3) |
| 44160442 | A/G | rs9900627 | Synonymous | S171S | 2 (28.6) |
| 44160589 | G/A | rs8556 | Synonymous | S122S | 5 (71.4) |

*HOXB13* variants identified in 2 Asian American samples

| Chrm 17 Location* | Ref Allele/Variant Allele | dbSNP ID | Variant Type | Substitution | # Individuals [N(%)] |
|---|---|---|---|---|---|
| 44157328 | T/C | rs79812861** | 3' UTR | NA | 2 (100) |
| 44158537 | C/T | rs11653611 | 3' UTR | NA | 2 (100) |
| 44160589 | G/A | rs8556 | Synonymous | S122S | 2 (100) |

*Location based on hg18 reference sequence

**These dbSNP ID numbers were not available at the completion of sequencing.

**Supplementary Table 2: Clinical features of genotyped samples.** All men studied described themselves as European descent and the sample sizes are variable due to missing data.

| University of Michigan | | | | |
|---|---|---|---|---|
| | Mean | St. Dev. | Median | Range |
| Age at Dx (yrs) (n=1130) | 52.2 | 6.3 | 52 | 27-77 |
| RP Gleason Grade (n=938) | 6.60 | 0.85 | 7 | 3-10 |
| | | | | |
| Johns Hopkins University | | | | |
| | Mean | St. Dev | Median | Range |
| Age at Dx (yrs) (n=3797) | 58.5 | 6.7 | 59 | 35-85 |
| RP Gleason Grade (n=3752) | 6.47 | 0.79 | 6 | 4-10 |

Abbreviations: RP = radical prostatectomy, Dx = diagnosis, St. Dev. = standard deviation

**Supplementary Table 3: Summary of Results Comparing Age at Diagnosis and Family History Strata to Controls**

| | | | | 1401 JHU Controls | | 2662 Controls (JHU,ESP,HapMap) | |
|---|---|---|---|---|---|---|---|
| | G84E Carriers n (%) | Non-Carriers n (%) | Carrier Frequency | p-value | Odds Ratio (95% CI) | p-value | Odds Ratio (95% CI) |
| FH+ (n = 2064) | 45 (2.2) | 2019 (97.8) | 2.2 | $1.8x10^{-9}$ | 31.2 (5.3,1253.3) | $1.7x10^{-12}$ | 14.8 (5.4,56.8) |
| FH- (n= 2410) | 19 (0.8) | 2391(99.2) | 0.79 | 0.0018 | 11.1 (1.8,461.7) | 0.0011 | 5.3 (1.8,21.4) |
| Age Dx ≤55 (n = 2130) | 46 (2.2) | 2084 (97.8) | 2.2 | $2.4x10^{-9}$ | 30.9 (5.3,1240.7) | $1.3x10^{-12}$ | 14.7(5.3,56.2) |
| Age Dx >55 (n = 2703) | 22 (0.8) | 2681 (99.2) | 0.81 | 0.0014 | 11.5 (1.9,473.5) | 0.00052 | 5.5 (1.8,21.8) |
| FH+ and Age Dx ≤55 (n = 1073) | 33 (3.1) | 1040 (96.9) | 3.1 | $1.6x10^{-11}$ | 44.3 (7.4,1792.6) | $1.7x10^{-14}$ | 21.1 (7.5,82.0) |
| FH+ and Age Dx >55 | 12 (1.2) | 993 (98.8) | 1.2 | 0.00022 | 16.9 (2.5,721.3) | $9.8x10^{-5}$ | 8.0 (2.4,34.2) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (n = 1005) | | | | | | | |
| FH- and Age Dx ≤55<br>(n = 953) | 10 (1.0) | 943 (99.0) | 1.0 | 0.00080 | 14.8 (2.1,642.8) | 0.00053 | 7.0 (2.0,30.8) |
| FH- and Age Dx >55<br>(n = 1456) | 9 (0.6) | 1447<br>(99.4) | 0.62 | 0.022 | 8.7 (1.2,381.3) | 0.017 | 4.1 (1.2,18.4) |

Abbreviations: Dx = diagnosis, FH = family history

**Supplementary Table 4: Case-Only Analyses of Clinical Characteristics in Combined UM-PCGG and JHU Cases by G84E Carrier Status**

| | G84E Carriers | | Non-Carriers | | |
|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | p-value |
| Age at Dx (yrs) | 52.9 | 0.9 | 57.1 | 0.1 | $7.4 \times 10^{-7}$ |
| RP Gleason Grade | 6.42 | 0.10 | 6.49 | 0.01 | 0.49 |
| RP Gleason Grade adjusted for Age at Dx* | 6.44 | 0.10 | 6.49 | 0.01 | 0.65 |

Abbreviations: RP = radical prostatectomy, Dx = diagnosis

**Supplement Figure 1**. **IHC staining for HOXB13 (Panels A, C, and E) and AMACR (Panels B and D) in benign (Panel A) and malignant prostate tissue (Panels B-E).** Note prominent staining of HOXB13 in nuclei of both normal luminal epithelial cells and cancer cells. Tumor-specific staining of AMACR is present in the cytoplasm of cancer cells. Method: Sections of FFPE tissue from a HOXB13 G84E carrier were stained with antibodies against HOXB13 (F-9, Santa Cruz Biotechnology) or AMACR (13H4, Dako North America Inc).